Ethanol extract of *Chondracanthus tenellus* (Harvey) Hommersand attenuates lipopolysaccharide−induced inflammatory and oxidative response by blocking the NF−κB, MAPKs, and PI3K/Akt signaling pathways

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ABSTRACT

Objective: To investigate whether the ethanol extract of *Chondracanthus tenellus* (Harvey) Hommersand, a type of red algae, could exhibit anti-inflammatory potential in lipopolysaccharide (LPS)-stimulated macrophages.

Methods: The ethanol extract of *Chondracanthus tenellus* was applied to 100 ng/mL LPS-stimulated RAW 264.7 cells, and cell viability, phagocytic ability, levels of pro-inflammatory factors, and the production of reactive oxygen species were measured. To identify the underlying mechanism of the ethanol extract of *Chondracanthus tenellus*, the expression of inflammation-regulated genes was estimated.

Results: The ethanol extract of *Chondracanthus tenellus* had no cytotoxic effect at concentrations below 300 μg/mL, and reduced the LPS-induced production of inflammatory mediators including nitric oxide (NO) and prostaglandin E_2. Furthermore, the extract markedly suppressed the expression of inducible NO synthase and cyclooxygenase-2, as well as the production of reactive oxygen species. The LPS-induced up-regulation of pro-inflammatory cytokines was attenuated by treatment with the ethanol extract of *Chondracanthus tenellus*, reducing their extracellular secretion. The *Chondracanthus tenellus* extract also inhibited LPS-mediated activation of nuclear factor-kappa B (NF-κB). In addition, the phosphorylation of mitogen activated protein kinases (MAPKs) and phosphatidylinositol 3 kinase (PI3K)/Akt was markedly increased by LPS, which was significantly abolished by the *Chondracanthus tenellus* extract.

Conclusions: Our findings indicate that the ethanol extract of *Chondracanthus tenellus* exhibited potential anti-inflammatory and antioxidant effects through downregulating the NF-κB, MAPKs, and PI3K/Akt signaling pathways in LPS stimulated RAW 264.7 macrophages.

KEYWORDS: *Chondracanthus tenellus*; Inflammation; ROS; NF-κB; RAW 264.7 cells

1. Introduction

A properly controlled inflammation is a biological response of the immune system, but uncontrolled excessive inflammation

contributes to chronic inflammatory diseases such as rheumatoid arthritis, diabetes, atherosclerosis, pulmonary fibrosis, chronic hepatitis, degenerative diseases, as well as various cancers[1–3]. Macrophages are potent innate immune cells that regulate immune homeostasis in the physiological and pathological stages. Lipopolysaccharide (LPS) is known to be a critical pathogenic stimulator in the innate immune system[4]. Accumulated evidence suggested that LPS stimulates local or systemic inflammatory response[4]. Among the pro-inflammatory mediators, nitric oxide (NO) and prostaglandin E_2 (PGE_2) are synthesized by inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2), respectively[5,6]. In LPS-stimulated macrophages, the expression of iNOS and COX-2 is closely correlated with the immoderate generation of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-10, and interferon-γ[5–8]. There is well known that LPS-mediated inflammatory response is activated by the toll-like receptor 4-mediated signaling pathways, including nuclear factor-kappa B (NF-κB), mitogen activated protein kinases (MAPKs), and phosphatidylinositol 3 kinase (PI3K)/Akt[4,9]. Meanwhile, reactive oxygen species (ROS) act as integral signaling molecules that regulate autoimmune response and immune-mediated inflammatory diseases[1,10]. In macrophages, ROS was stimulated by LPS, promoting the inflammatory response, and overgenerated inflammatory factors may accelerate to enhance ROS generation[1,11].

Recently, there has been increasing interest in using marine species as a resource for suppressing various inflammatory and oxidative diseases[12–14]. Among them, seaweed has great potential to develop as a preventive agent for these diseases because of the abundance of physiologically active substances with various pharmacological actions[14–16]. For example, several red algae extracts, including *Sarcodia ceylanica*, *Porphyra dioica*, *Gelidium elegans*, and *Chondrus crispus*, have been reported to exhibit the anti-inflammatory effect in LPS-stimulated macrophages[17–21]. In addition, edible red algae *Gelidium elegans* extract has been reported to attenuate lipid accumulation and the generation of ROS and reactive nitrogen species in 3T3-L1 preadipocytes and RAW 264.7 macrophages[18,22]. Moreover, Wijesinghe *et al.*[23] reported that the anti-inflammatory efficacy of a bioactive substance isolated from *Laurencia snackeyi*, a red seaweed was associated with inhibition of oxidative stress in a zebrafish embryo. *Chondracanthus tenellus* (*C. tenellus*) (Harvey) Hommersand is a species of red algae distributed throughout the coasts of East Asia and has long been consumed as an edible seaweed. Although *C. tenellus* is known to reduce ultraviolet B-induced cellular damage by scavenging ROS in human keratinocytes[24], research on its pharmacological action has been limited to date. Therefore, in this study, the effect of the ethanol extract of *C. tenellus* on LPS-stimulated RAW 264.7 macrophage cells was investigated and the related mechanisms were elucidated.

2. Materials and methods

2.1. Preparation of the ethanol extract of C. tenellus

C. tenellus was collected from offshore Jeju Island, Republic of Korea in March 2017, the ethanol extract of *C. tenellus* used in the current study was provided by Dr. DS Lee from National Marine Biodiversity Institute (Seocheon, Republic of Korea). A voucher specimen of the fruits (WECU-17-6) was deposited in the Department of Biochemistry, Dongeui University College of Korean Medicine (Busan, Republic of Korea). In brief, dried *C. tenellus* was extracted five times with 70% ethyl alcohol (1:10 *w/v*) at 1 h interval by sonication. Subsequently, the extract was evaporated in vacuo, and then dissolved in dimethyl-sulfoxide (DMSO, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The concentration of DMSO used in this study below 0.05% did not affect cytotoxicity on RAW 264.7 cells.

2.2. Cell culture

The RAW 264.7 cells (ATCC® TIB-71™) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin, in atmosphere of 5% CO_2 at 37 ℃. All reagents for cell culture were obtained from WelGENE Inc. (Daegu, Republic of Korea).

2.3. Cell viability

To determine cytotoxicity of the ethanol extract of *C. tenellus* in RAW 264.7 cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich Chemical Co.) reduction assay was conducted[25]. Briefly, the cells were seeded in 96-well plates ($1×10^4$ cells/wells), and were treated with various concentrations of the ethanol extract of *C. tenellus* or pre-treatment with the ethanol extract of *C. tenellus* for 1 h before exposure to 100 ng/mL LPS (Sigma-Aldrich Chemical Co.) for 24 h. The cells were incubated with 500 μg/mL of MTT solution for 3 h. Subsequently, the cells were lysed with DMSO, and the absorbance was measured using a microplate reader (Dynatech Laboratories, Chantilly, VA, USA). The cell morphology was observed under a microscope (Carl Zeiss, Oberkochen, Germany).

2.4. Phagocytic activity

The phagocytosis was analyzed using a commercial phagocytosis assay kit (Cayman Chemical, Ann Arbor, MI, USA), as previously described[26]. The extent of phagocytosis was determined using Cell Lab Quanta SC flow cytometry (Beckman Coulter, Brea, CA, USA).

2.5. The levels of NO, PGE$_2$, and cytokines

The NO level in the supernatants was measured using Griess reagent (Sigma-Aldrich Chemical Co.)[27]. The levels of PGE$_2$ and cytokines were assayed using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions, as previously described[28].

2.6. Western blotting analysis

The total proteins were lysed with lysis buffer as previously described[28], and proteins from the nucleus and cytoplasm were extracted using nuclear extraction reagents (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred into polyvinylidene difluoride membranes. The protein-transferred membranes were blocked and then reacted with primary antibodies (Table 1) overnight at 4 ℃. Subsequently, the membranes were incubated with secondary antibodies for 2 h at room temperature. Actin and lamin B were used as an internal control for cytosol and nucleus, respectively. Protein bands were visualized *via* X-ray film. Densitometric analysis of the bands was performed using the ImageJ® software (v1.48, NIH, Bethesda, MD).

2.7. Immunofluorescence staining for NF–κB

The cells were pre-treated with 300 μg/mL of the ethanol extract of *C. tenellus* for 1 h and then stimulated with or without 100 ng/mL LPS for 1 h. The cells were fixed, blocked, and then probed with anti-NF-κB at 4 ℃ overnight. The cells were reacted with the goat anti-rabbit IgG cross-absorbed secondary antibody conjugated to Alexa Fluor 594 (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h.

The cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.), which is a nucleus-specific dye, and observed under a fluorescence microscope (Carl Zeiss).

2.8. Assessment of ROS levels

Cells (3×10^5 cells/mL) were cultured with the ethanol extract of *C. tenellus* and/or LPS for 1 h and were stained with 10 μM DCF-DA for 15 min. The stained cells were visualized under a fluorescence microscope and analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA) as previously described[29].

2.9. Statistical analysis

All data were expressed as mean ± standard deviation (SD) of at least triplicate experiments and analyzed with GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data were compared with one-way analysis of variance following by Tukey's *post hoc* test. Differences were considered statistically significant if the P value was < 0.05.

3. Results

3.1. Cytotoxic effect of the ethanol extract of C. tenellus

Figure 1A shows that the ethanol extract of *C. tenellus* had no cytotoxic effect on RAW 264.7 cells at concentrations below 300 μg/mL. However, remarkable cytotoxicity was observed in over 400 μg/mL-treated cells. Meanwhile, no cytotoxicity was observed when 100 ng/mL LPS-treated cells were treated with the ethanol extract of *C. tenellus* at concentrations of no more than 300 μg/mL. Moreover, a feature of activated macrophages such as polygonal spindle-shaped pseudopodia was

Table 1. List of primary antibodies used for Western blotting analysis in the study.

| Antibody	Manufacturer	Catalog No.	Dilution
iNOS	BD Biosciences (San Jose, CA, USA)	610328	1:1 000
COX-2	Cayman Chemical Company (Ann Arbor, MI, USA)	160126	1:500
TNF-α	Cell Signaling Technology (Beverly, MA, USA)	#3707	1:1 000
IL-6	Santa Cruz Biotechnology, Inc.	sc-28343	1:1 000
IL-10	R&D Systems Inc.	MAB217-SP	1:1 000
NF-κB	Santa Cruz Biotechnology, Inc.	Sc-8008	1:500
IκB-α	Cell Signaling Technology	#9242	1:500
Laminin B	Santa Cruz Biotechnology, Inc.	sc-374015	1:1 000
p-p38	Cell Signaling Technology	#9211	1:1 000
p38	Santa Cruz Biotechnology, Inc.	sc-535	1:1 000
p-ERK	Cell Signaling Technology	#9106	1:1 000
ERK	Santa Cruz Biotechnology, Inc.	sc-154	1:1 000
p-JNK	Cell Signaling Technology	#9255	1:1 000
JNK	Cell Signaling Technology	#9252S	1:1 000
p-PI3K	Cell Signaling Technology	#4228	1:1 000
PI3K	Cell Signaling Technology	#4257	1:1 000
p-Akt	Santa Cruz Biotechnology, Inc.	sc-101629	1:1 000
Akt	Santa Cruz Biotechnology, Inc.	sc-8312	1:1 000
Actin	Santa Cruz Biotechnology, Inc.	sc-47778	1:1 000

A

B

C
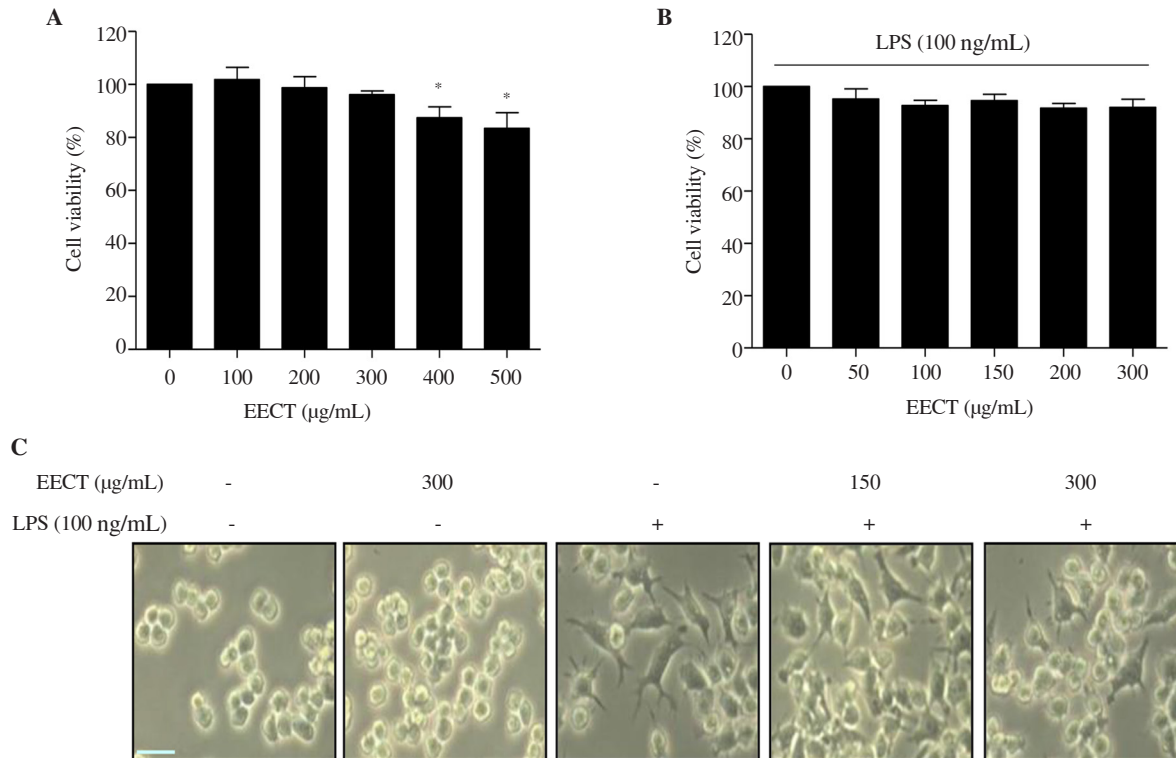
Figure 1. Effect of EECT on the cytotoxicity of RAW 264.7 macrophages. (A) Cytotoxic effect of different concentrations of EECT on RAW 264.7 cells. (B) Cell viability of LPS-stimulated cells with or without EECT treatment. Each value indicates mean ± SD (n=3). * indicates significant difference from untreated cells at $P < 0.05$. (C) The cell morphology was observed using a microscope. Scale bar: 200 μm. (magnification: × 200) EECT: the ethanol extract of *Chondracanthus tenellus*. LPS: lipopolysaccharide.
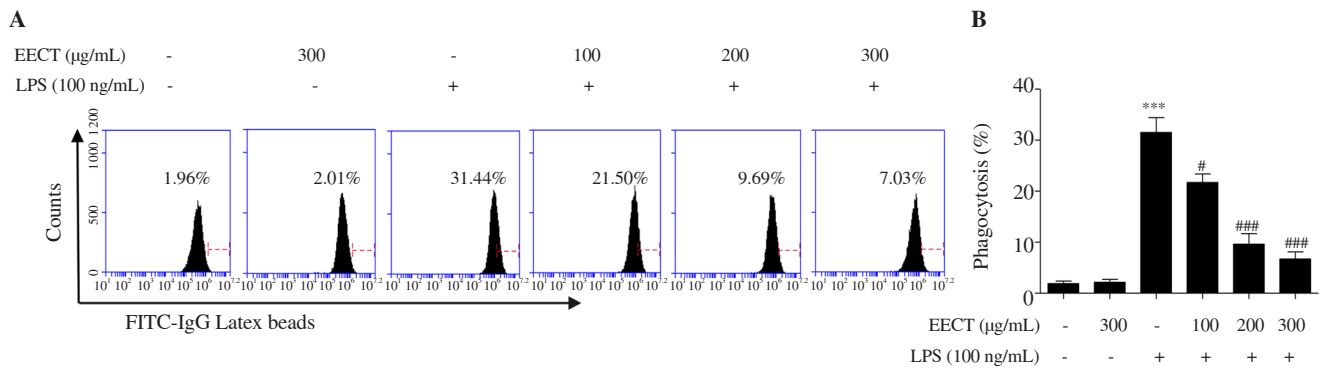
A

B

Figure 2. Effect of EECT on cell morphology and phagocytosis in LPS-stimulated RAW 264.7 macrophages. (A and B) The phagocytic uptake of FITC-labeled dextran was measured by flow cytometry. (A) The representative plots. (B) The histogram results of phagocytic uptake of FITC-dextran. Data are expressed as mean ± SD (n=3). *** indicates significant difference from untreated cells at $P < 0.001$. # and ### indicate significant difference from LPS-stimulated cells at $P < 0.05$ and $P < 0.001$, respectively.

verified in LPS-stimulated cells, whereas the original round shape largely existed in the presence of the ethanol extract of *C. tenellus* (Figure 1C).

3.2. Prevention of phagocytosis by the ethanol extract of C. tenellus in LPS–stimulated RAW 264.7 macrophages

To evaluate the effect of the ethanol extract of *C. tenellus* on phagocytosis that is one of the key features of abnormally activated macrophages, we determined phagocytic activity using a commercial analysis kit. Figures 2A and B show that LPS greatly promoted phagocytosis in RAW 264.7 cells, whereas LPS-induced phagocytosis was markedly attenuated by the ethanol extract of *C. tenellus* in a concentration-dependent manner.

3.3. Inhibition of LPS–induced inflammatory mediator release by the ethanol extract of C. tenellus in RAW 264.7 macrophages
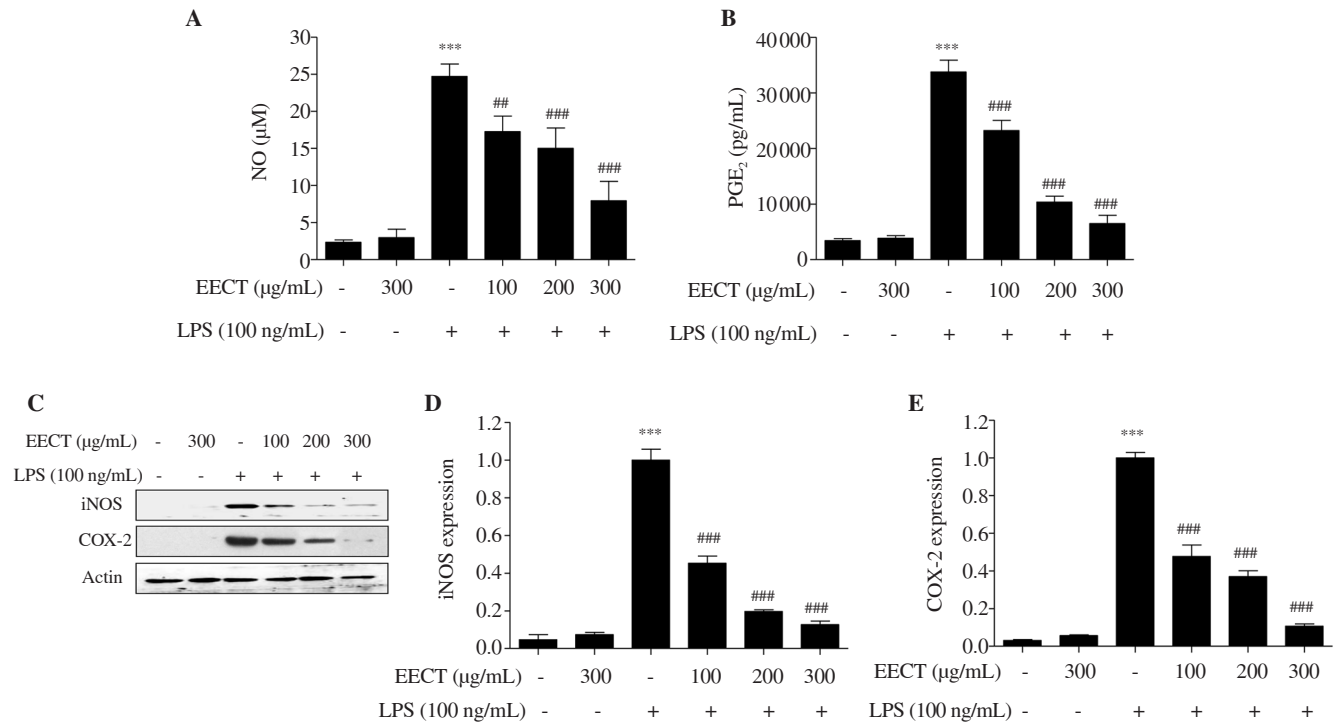
Figures 3A and B show that LPS treatment greatly promoted

Figure 3. Effect of EECT on the production of pro-inflammatory mediators in LPS-stimulated RAW 264.7 macrophages. (A) NO concentration was measured by Griess reaction. (B) PGE_2 concentration was determined using a commercial ELISA kit. (C) The expression of iNOS and COX-2 was determined by Western blotting. (D and E) Band densities were quantified using ImageJ and normalized against actin. Data are expressed as mean ± SD (n=3). *** indicates significant difference from untreated cells at $P < 0.001$. ## and ### indicate significant difference from LPS-stimulated cells at $P < 0.01$ and $P < 0.001$, respectively. NO: nitric oxide, PGE_2: prostaglandin E_2, iNOS: inducible NO synthase, COX-2: cyclooxygenase-2.
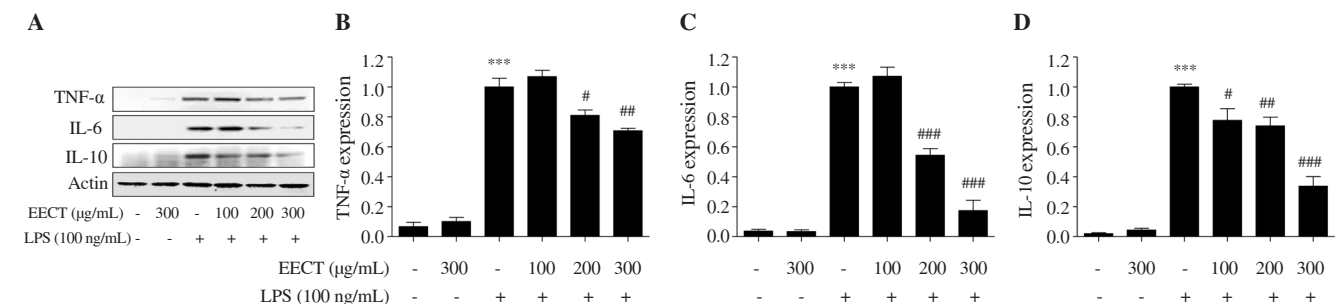

Figure 4. Effect of EECT on the production pro-inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages. (A) The expression of cytokines (TNF-α, IL-6 and IL-10) was measured by Western blotting. (B-D) Bands were quantified using ImageJ and normalized against actin. Data are expressed as mean ± SD (n=3). *** indicates significant difference from untreated cells at $P < 0.001$. #, ## and ### indicate significant difference from LPS-stimulated cells at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.
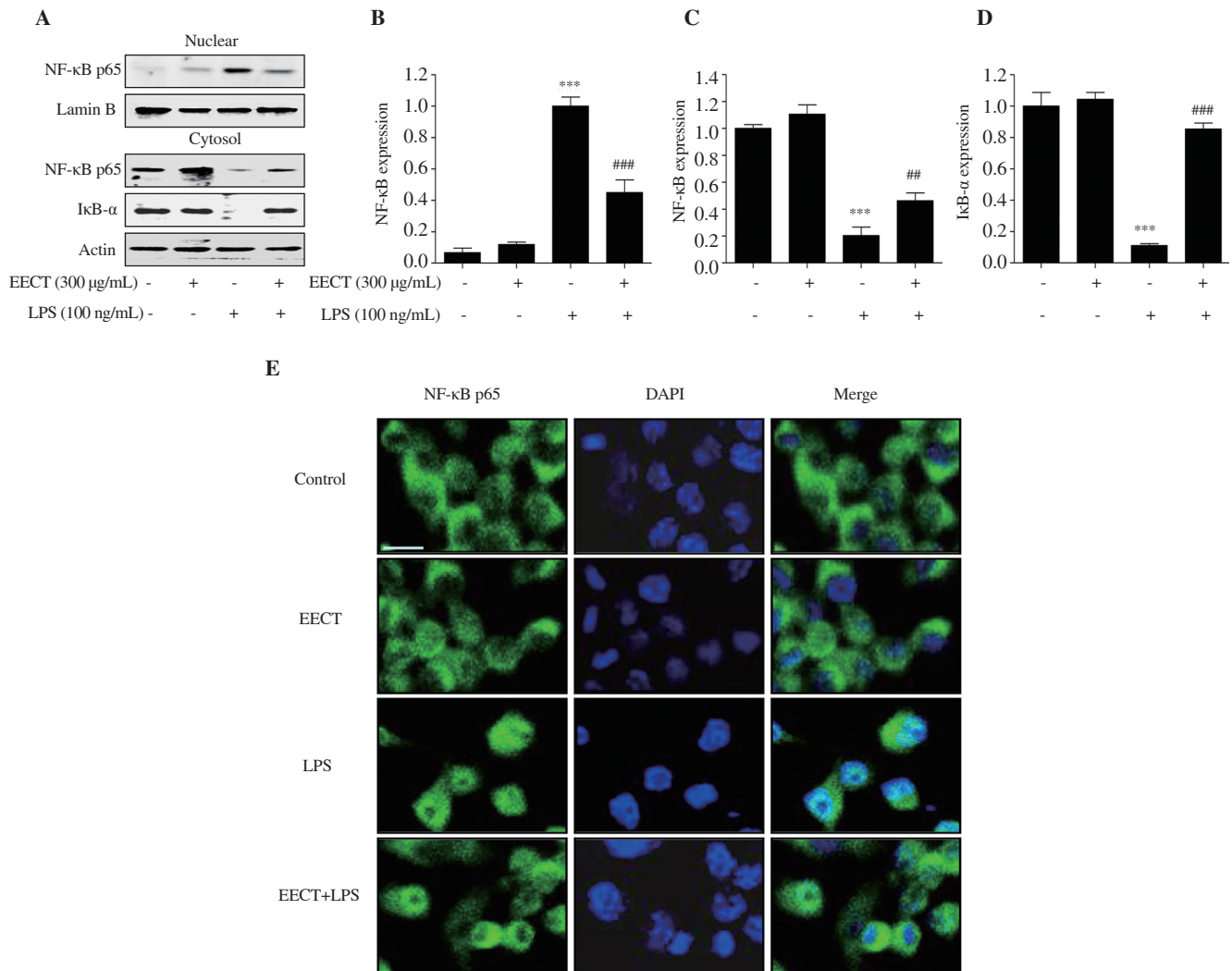
Figure 5. Effect of EECT on the activation of the NF-κB signaling pathway in LPS-stimulated RAW 264.7 macrophages. (A) The expression of NF-κB and IκB-α in the cytoplasm and nucleus. Bands were quantified using ImageJ and normalized against lamin B (B) or actin (C and D), and the ratio was determined. Data are expressed as mean ± SD (n=3). (E) The representative images of immunofluorescence staining with NF-κB p65. Green fluorescence indicates the localization of NF-κB p65. DAPI was used for nuclear counterstaining (blue). Scale bar: 75 μm. *** indicates significant difference from untreated cells at $P <$ 0.001. ## and ### indicate significant difference from LPS-stimulated cells at $P <$ 0.01 and $P <$ 0.001, respectively.

the levels of NO and PGE$_2$ whereas this increment was markedly inhibited by the ethanol extract of *C. tenellus*. Furthermore, according to the Western blotting results, the expression of iNOS and COX-2 was up-regulated by LPS, which was substantially down-regulated by pre-treatment of the ethanol extract of *C. tenellus* (Figures 3C-E).

3.4. Inhibition of production and expression of pro−inflammatory cytokines by the ethanol extract of C. tenellus in RAW 264.7 macrophages

The levels of TNF-α, IL-6, and IL-10 were significantly enhanced by LPS stimulation. However, the increment of these pro-inflammatory cytokines by LPS was markedly reduced in the presence of the ethanol extract of *C. tenellus*. Moreover, LPS markedly elevated the expression of TNF-α, IL-6, and IL-10, which was suppressed by the ethanol extract of *C. tenellus* (Figures 4A-D).

3.5. Amelioration of nuclear translocation of NF−κB by the ethanol extract of C. tenellus in LPS−stimulated RAW 264.7 macrophages

Next, we evaluated the effect of the ethanol extract of *C. tenellus* on LPS-mediated activation of NF-κB that is a critical transcriptional factor controlling inflammatory response. Figures 5A-D show that the expressions of NF-κB in the nucleus and inhibitor of nuclear factor-κB (IκB)-α in the cytoplasm were significantly enhanced and decreased by LPS stimulation, respectively. However, this activation of NF-κB by LPS was markedly reversed in the presence of the ethanol extract of *C. tenellus*. This result corresponded with the result from fluorescence staining that the increase in fluorescence intensity of NF-κB p65 observed in the nuclei of LPS-treated cells was markedly reduced by pre-treatment with the ethanol extract of *C. tenellus* (Figure 5E).
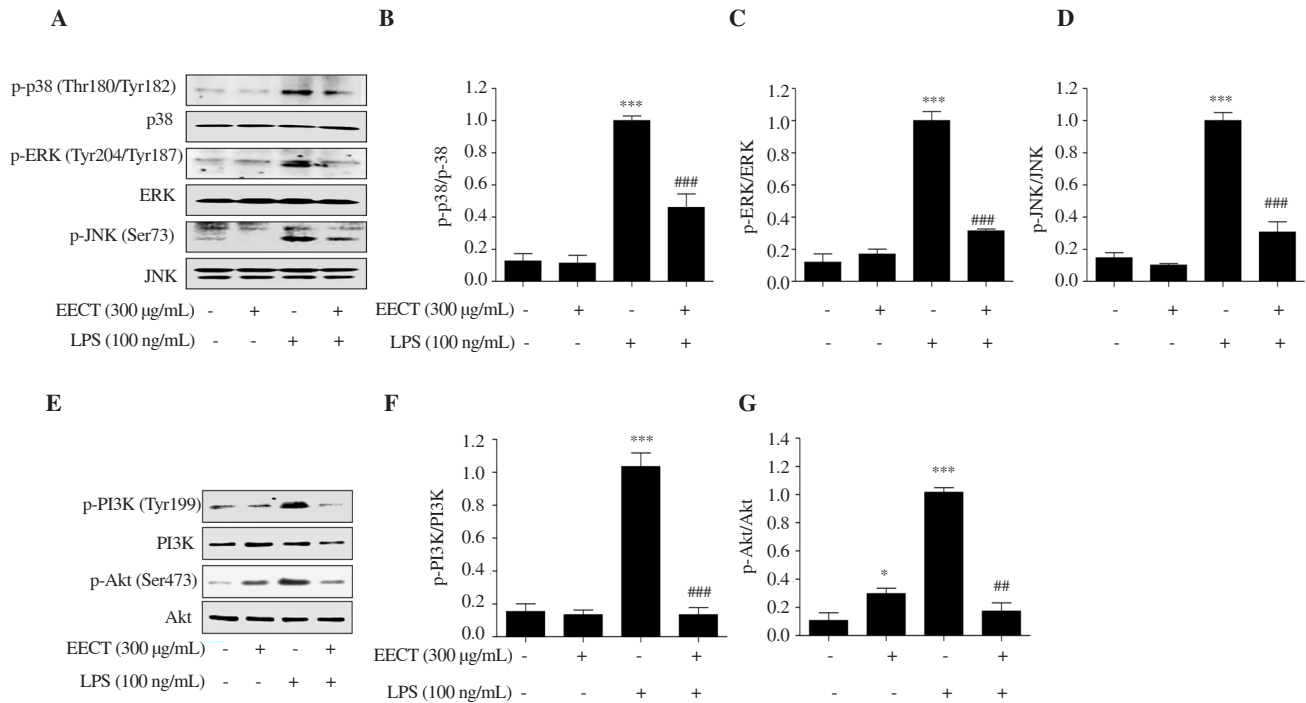
Figure 6. Effect of EECT on the activation of the MAPKs and PI3K/Akt signaling pathways in LPS-stimulated RAW 264.7 macrophages. (A and E) Expression of proteins involved in the MAPKs and PI3K/Akt signaling pathways. (B, C, D, F and G) Bands were quantified using ImageJ and normalized against lamin B or actin. Data are presented as mean ± SD (n=3). * and *** indicate significant difference from untreated cells at $P < 0.05$ and $P < 0.001$, respectively. ## and ### indicate significant difference from LPS-stimulated cells at $P < 0.01$ and $P < 0.001$, respectively.

3.6. Inhibitory effect of the ethanol extract of C. tenellus on the activation of MAPK and PI3K/Akt signaling pathways in LPS−stimulated RAW 264.7 macrophages

To assess the regulation of the upstream signaling cascade associated with inhibition of NF-κB activity, we determined a family of conserved enzymes involved in the MAPKs and PI3K/Akt signaling pathways. Figures 6A-D show that the phosphorylation levels of three kinases belonging to MAPKs, including p38 MAPK, extracellular signal-regulated kinase (ERK), and c-Jun *N*-terminal kinase (JNK), were upregulated in LPS-stimulated cells compared with the control group. In addition, the expression of the phosphorylated form of PI3K and its downstream gene, *Akt*, was increased by LPS treatment (Figures 6E-G). These results indicate that both the MAPKs and PI3K/Akt signaling pathways were activated in LPS-treated cells. However, LPS-induced phosphorylation of these enzymes was completely attenuated in the presence of the ethanol extract of *C. tenellus*.

3.7. Reduction of LPS−mediated ROS generation by the ethanol extract of C. tenellus in RAW 264.7 macrophages

To evaluate the effect of the ethanol extract of *C. tenellus* on LPS-stimulated oxidative stress that plays a critical role in the activation of macrophages, we measured the intracellular oxidative stress levels. Figures 7A and B show that the levels of intracellular ROS

contents were increased with the stimulation of LPS, but the ethanol extract of *C. tenellus* significantly reduced DCF-DA-positive cells in LPS-stimulated RAW 264.7 cells. As shown in Figure 7C, the enhancement in the fluorescence intensity of DCF-DA by LPS stimulation was greatly weakened by the ethanol extract of *C. tenellus*, which was consistent with the result of flow cytometry.

4. Discussion

To estimate the anti-inflammatory efficacy of the ethanol extract of *C. tenellus*, we evaluated its effect on the production of NO and PGE_2[5,7,30,31]. Among them, NO controls normal physiological functions, but under pathological conditions, the excessive NO produced by iNOS triggers the inflammatory response and oxidative stress[30,32]. PGE_2 is mainly synthesized by COX-2, and excessive PGE_2 acts as an inflammatory key mediator[5,7,30,31]. Therefore, suppressors against overproduction of these inflammatory mediators could be considered as potential therapeutic agents for inflammatory treatment.

In this regard, our findings showed that the section of NO and PGE_2 and the expression of iNOS and COX-2 were markedly increased by LPS, but up-regulation of these inflammatory mediators was greatly decreased in the presence of the ethanol extract of *C. tenellus*. Macrophages produce several pro-inflammatory cytokines that are required in the initiation and improvement of the inflammatory
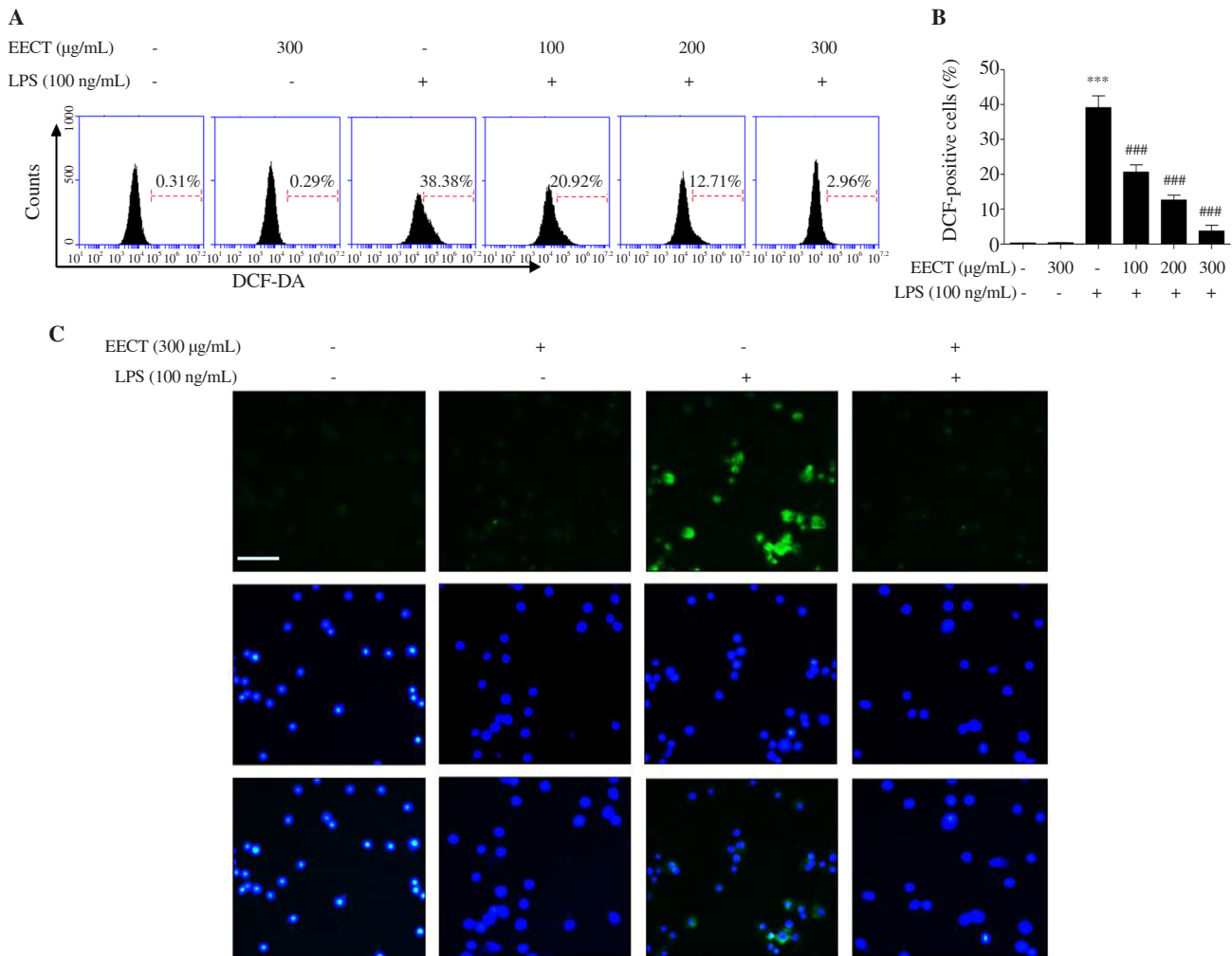
Figure 7. Inhibition of ROS generation by EECT in LPS-stimulated RAW 264.7 macrophages. (A) The representative histograms of DCF-DA-stained cells. (B) The percentages of DCF-DA-stained cells. Data are presented as mean ± SD (*n*=3). (C) The representative fluorescence micrographs depicting ROS generation. Green fluorescence indicates the intensity of ROS generation. Nuclei are visualized by DAPI staining (blue). Scale bar: 200 μm. *** indicates significant difference from untreated cells at $P < 0.001$. ### indicates significant difference from LPS-stimulated cells at $P < 0.001$.

response and promote the inflammatory response by vicious cycle[5,7,8,32–35]. Therefore, the level of pro-inflammatory cytokines has been considered as an indicator to assess anti-inflammatory efficacy in macrophages. In this study, our results indicated that the ethanol extract of *C. tenellus* decreased the secretion of TNF-α, IL-6, and IL-10 in LPS-stimulated RAW 264.7 cells by suppressing their expressions.

NF-κB plays a key transcriptional factor involved in the control of inducible anti-inflammatory enzymes and cytokines in LPS-activated macrophages[4,9]. Typically, NF-κB is bound to the inhibitory subunit IκB-α and remains inactive in the cytoplasm. When IκB-α is phosphorylated and degraded by inflammatory stimuli such as LPS, NF-κB migrates to the nucleus and triggers the transcriptional activity of inflammation-inducing cytokines and catabolic enzymes including iNOS and COX-2[36,37]. Therefore, the efficacy of the ethanol extract of *C. tenellus* on LPS-induced NF-κB activation was investigated because the inhibition of the activity of NF-κB could be effective against inflammation. The current results showed that the translocation of NF-κB from the cytoplasm into the nucleus and

the degradation of IκB-α were increased in LPS-treated RAW 264.7 cells, but the ethanol extract of *C. tenellus* effectively blocked the nuclear translocation of NF-κB and the degradation of IκB-α, an essential step for NF-κB activation. Therefore, the inhibitory effect of the ethanol extract of *C. tenellus* on the increased expression of pro-inflammatory enzymes and cytokines following LPS stimulation can be ascribed to the suppression of the nuclear translocation of NF-κB.

Accumulated evidence clearly shows that activation of NF-κB is involved in the activation of various intracellular upstream signaling molecules[4,38]. Among them, activated kinases belonging to the MAPKs and PI3K/Akt signaling pathways phosphorylate IκB for degradation through the ubiquitin-proteasome system, eventually enhancing NF-κB release and translocation from the cytoplasm to the nucleus[36,37]. Therefore, targeting the inactivation of the MAPKs and PI3K/Akt signaling pathways may be effective in treating inflammatory diseases. In this study, LPS-induced phosphorylation of p38 MAPK, ERK, and JNK belonging to MAPKs was remarkably down-regulated by pre-treatment of the ethanol extract of *C. tenellus*.

We further proved that LPS-stimulated phosphorylation of Akt and its upstream kinase, PI3K, was strongly inhibited by exposure to the ethanol extract of *C. tenellus*. These results indicate that the blocking effect of NF-κB by the ethanol extract of *C. tenellus* was at least involved in the obstruction of the MAPKs and PI3K/Akt signaling pathways. In addition, these observations support the reason for the reduction in pro-inflammatory cytokines and catabolites in the supernatant of the ethanol extract of *C. tenellus*-treated cells, similar to the actions of other anti-inflammatory agents reported in many previous studies[39–43]. However, our results suggest that the real targets of the ethanol extract of *C. tenellus* may be other upstream kinases other than MAPKs and PI3K/Akt, and questions about this await further research.

As noted in many previous studies, excess ROS accumulation can cause oxidative damage to cellular macromolecules, including nucleic acids, proteins, and lipids, and organelles[1,10]. There is well known that ROS result in the activation of macrophages, and are involved in initiating and promoting inflammatory response by pro-inflammatory mediators and cytokines[1,10,11]. In this study, the ethanol extract of *C. tenellus* substantially suppressed LPS-induced ROS formation in RAW 264.7 macrophages. This result supported the application of the ethanol extract of *C. tenellus* as an antioxidant for the management of oxidative stress associated with inflammatory responses.

In this study, the anti-inflammatory and antioxidant potential of the ethanol extract of *C. tenellus* was demonstrated in an LPS-treated RAW 264.7 macrophage model. According to our results, the morphological changes and phagocytic activation of RAW 264.7 cells by LPS were significantly suppressed by the ethanol extract of *C. tenellus*. Furthermore, the LPS-induced overexpression of pro-inflammatory enzymes including iNOS and COX-2 and overproduction of inflammatory cytokines such as TNF-α, IL-6, and IL10 were significantly attenuated in the presence of the ethanol extract of *C. tenellus*. These results suggested that the ethanol extract of *C. tenellus* exhibits potential suppressive effect on LPS-induced activation and inflammation of macrophages. In addition, the ethanol extract of *C. tenellus* attenuated the accumulation of ROS and suppressed the activation of NF-κB, MAPKs, and PI3K/Akt signaling pathways, indicating that the anti-inflammatory and antioxidant effects of the ethanol extract of *C. tenellus* on LPS stimulation are related to the inactivation of these signaling pathways in RAW 264.7 macrophages. However, further studies are needed to explore the role of cellular signaling pathways that may be involved in the antioxidant activity of the ethanol extract of *C. tenellus* and to determine a direct relationship with its anti-inflammatory efficacy. It is also necessary to verify the efficacy of the ethanol extract of *C. tenellus* through animal experiments and to identify the active ingredients contained in the ethanol extract of *C. tenellus*.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Authors' contributions

Collecting data, drafting the manuscript and critical revision of the manuscript were done by DHK, CP, HL, SHH and YHC. YHC, CP and HJH designed the study. Data analysis and interpretation were done by GYK, HJC, HSK and HJH. In addition, YHC was responsible for supervision, as well as SK and HJH contributed to project administration and funding acquisition.

References

[1] Liu Z, Ren Z, Zhang J, Chuang CC, Kandaswamy E, Zhou T, et al. Role of ROS and nutritional antioxidants in human diseases. *Front Physiol* 2018; **9**: 477.

[2] Aksentijevich M, Lateef SS, Anzenberg P, Dey AK, Mehta NN. Chronic inflammation, cardiometabolic diseases and effects of treatment: Psoriasis as a human model. *Trends Cardiovasc Med* 2020; **30**: 472-478.

[3] Semb AG, Ikdahl E, Wibetoe G, Crowson C, Rollefstad S. Atherosclerotic cardiovascular disease prevention in rheumatoid arthritis. *Nat Rev Rheumatol* 2020; **16**: 361-379.

[4] Hernandez A, Patil NK, Stothers CL, Luan L, McBride MA, Owen AM, et al. Immunobiology and application of toll-like receptor 4 agonists to augment host resistance to infection. *Pharmacol Res* 2019; **150**: 104502.

[5] Soufli I, Toumi R, Rafa H, Touil-Boukoffa C. Overview of cytokines and nitric oxide involvement in immuno-pathogenesis of inflammatory bowel diseases. *World J Gastrointest Pharmacol Ther* 2016; **7**: 353-360.

[6] Förstermann U, Sessa WC. Nitric oxide synthases: Regulation and function. *Eur Heart J* 2012; **33**: 829-837.

[7] Aleem D, Tohid H. Pro-inflammatory cytokines, biomarkers, genetics and the immune system: A mechanistic approach of depression and psoriasis. *Rev Colomb Psiquiatr* 2018; **47**: 177-186.

[8] Wyns H, Plessers E, De Backer P, Meyer E, Croubels S. *In vivo* porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. *Vet Immunol Immunopathol* 2015; **166**: 58-69.

[9] Doyle SL, O'Neill LA. Toll-like receptors: From the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochem Pharmacol* 2006; **72**: 1102-1113.

[10] Bjørn ME, Hasselbalch HC. The role of reactive oxygen species in myelofibrosis and related neoplasms. *Mediators Inflamm* 2015; **2015**: 648090.

[11] Mills EL, O'Neill LA. Reprogramming mitochondrial metabolism in macrophages as an anti-inflammatory signal. *Eur J Immunol* 2016; **46**: 13-21.

[12] Dhakal D, Pokhrel AR, Shrestha B, Sohng JK. Marine rare

actinobacteria: Isolation, characterization, and strategies for harnessing bioactive compounds. *Front Microbiol* 2017; **8**: 1106.

[13]Barbalace MC, Malaguti M, Giusti L, Lucacchini A, Hrelia S, Angeloni C. Anti-inflammatory activities of marine algae in neurodegenerative diseases. *Int J Mol Sci* 2019; **20**: 3061.

[14]Pradhan B, Nayak R, Patra S, Jit BP, Ragusa A, Jena M. Bioactive metabolites from marine algae as potent pharmacophores against oxidative stress-associated human diseases: A comprehensive review. *Molecules* 2020; **26**: 37.

[15]Juárez-Portilla C, Olivares-Bañuelos T, Molina-Jiménez T, Sánchez-Salcedo JA, Moral DID, Meza-Menchaca T, et al. Seaweeds-derived compounds modulating effects on signal transduction pathways: A systematic review. *Phytomedicine* 2019; **63**: 153016.

[16]Jacobsen C, Sørensen AM, Holdt SL, Akoh CC, Hermund DB. Source, extraction, characterization, and applications of novel antioxidants from seaweed. *Annu Rev Food Sci Technol* 2019; **10**: 541-568.

[17]Kazłowska K, Hsu T, Hou CC, Yang WC, Tsai GJ. Anti-inflammatory properties of phenolic compounds and crude extract from *Porphyra dentata*. *J Ethnopharmacol* 2010; **128**: 123-130.

[18]Jeon HJ, Seo MJ, Choi HS, Lee OH, Lee BY. *Gelidium elegans*, an edible red seaweed, and hesperidin inhibit lipid accumulation and production of reactive oxygen species and reactive nitrogen species in 3T3-L1 and RAW264.7 cells. *Phytother Res* 2014; **28**: 1701-1709.

[19]Robertson RC, Guihéneuf F, Bahar B, Schmid M, Stengel DB, Fitzgerald GF, et al. The anti-inflammatory effect of algae-derived lipid extracts on lipopolysaccharide (LPS)-stimulated human THP-1 macrophages. *Mar Drugs* 2015; **13**: 5402-5424.

[20]Shih CC, Hwang HR, Chang CI, Su HM, Chen PC, Kuo HM, et al. Anti-inflammatory and antinociceptive effects of ethyl acetate fraction of an edible red macroalgae *Sarcodia ceylanica*. *Int J Mol Sci* 2017; **18**: 2437.

[21]da Costa E, Melo T, Moreira AS, Bernardo C, Helguero L, Ferreira I, et al. Valorization of lipids from *Gracilaria* sp. through lipidomics and decoding of antiproliferative and anti-inflammatory activity. *Mar Drugs* 2017; **15**: 62.

[22]Jeon HJ, Choi HS, Lee OH, Jeon YJ, Lee BY. Inhibition of reactive oxygen species (ROS) and nitric oxide (NO) by *Gelidium elegans* using alternative drying and extraction conditions in 3T3-L1 and RAW 264.7 cells. *Prev Nutr Food Sci* 2012; **17**: 122-128.

[23]Wijesinghe WA, Kim EA, Kang MC, Lee WW, Lee HS, Vairappan CS, et al. Assessment of anti-inflammatory effect of 5beta-hydroxypalisadin B isolated from red seaweed *Laurencia snackeyi* in zebrafish embryo *in vivo* model. *Environ Toxicol Pharmacol* 2014; **37**: 110-117.

[24]Piao MJ, Hyun YJ, Oh TH, Kang HK, Yoo ES, Koh YS, et al. *Chondracanthus tenellus* (Harvey) hommersand extract protects the human keratinocyte cell line by blocking free radicals and UVB radiation-induced cell damage. *In Vitro Cell Dev Biol Anim* 2012; **48**: 666-674.

[25]Choi YH. Trans-cinnamaldehyde protects C2C12 myoblasts from DNA damage, mitochondrial dysfunction and apoptosis caused by oxidative stress through inhibiting ROS production. *Genes Genomics* 2021; **43**(4): 303-312.

[26]Kwon DH, Lee H, Park C, Hong SH, Hong SH, Kim GY, et al. Glutathione induced immune-stimulatory activity by promoting M1-like macrophages polarization *via* potential ROS scavenging capacity. *Antioxidants (Basel)* 2019; **8**: 413.

[27]Chae BS. Effect of low-dose corticosterone pretreatment on the production of inflammatory mediators in super-low-dose LPS-primed immune cells. *Toxicol Res* 2020; **37**: 47-57.

[28]Park S, Kim M, Hong Y, Lee H, Tran Q, Kim C, et al. Myristoylated TMEM39AS41, a cell-permeable peptide, causes lung cancer cell death. *Toxicol Res* 2020; **36**: 123-130.

[29]Hwangbo H, Kim SY, Lee H, Park SH, Hong SH, Park C, et al. Auranofin enhances sulforaphane-mediated apoptosis in hepatocellular carcinoma Hep3B cells through inactivation of the PI3K/Akt signaling pathway. *Biomol Ther (Seoul)* 2020; **28**: 443-455.

[30]Saini R, Singh S. Inducible nitric oxide synthase: An asset to neutrophils. *J Leukoc Biol* 2019; **105**: 49-61.

[31]Yao C, Narumiya S. Prostaglandin-cytokine crosstalk in chronic inflammation. *Br J Pharmacol* 2019; **176**: 337-354.

[32]Wang T, He C, Yu X. Pro-inflammatory cytokines: New potential therapeutic targets for obesity-related bone disorders. *Curr Drug Targets* 2017; **18**: 1664-1675.

[33]Hu F, Lou N, Jiao J, Guo F, Xiang H, Shang D. Macrophages in pancreatitis: Mechanisms and therapeutic potential. *Biomed Pharmacother* 2020; **131**: 110693.

[34]Zhang H, Cai D, Bai X. Macrophages regulate the progression of osteoarthritis. *Osteoarthritis Cartilage* 2020; **28**(5): 555-561.

[35]Popa C, Netea MG, van Riel PL, van der Meer JW, Stalenhoef AF. The role of TNF-alpha in chronic inflammatory conditions, intermediary metabolism, and cardiovascular risk. *J Lipid Res* 2007; **48**: 751-762.

[36]Rigoglou S, Papavassiliou AG. The NF-kappaB signalling pathway in osteoarthritis. *Int J Biochem Cell Biol* 2013; **45**: 2580-2584.

[37]Schuliga M. NF-kappaB signaling in chronic inflammatory airway disease. *Biomolecules* 2015; **5**: 1266-1283.

[38]Wei J, Feng J. Signaling pathways associated with inflammatory bowel disease. *Recent Pat Inflamm Allergy Drug Discov* 2010; **4**: 105-117.

[39]Yan T, Yu X, Sun X, Meng D, Jia JM. A new steroidal saponin, furotrilliumoside from *Trillium tschonoskii* inhibits lipopolysaccharide-induced inflammation in Raw264.7 cells by targeting PI3K/Akt, MARK and Nrf2/HO-1 pathways. *Fitoterapia* 2016; **115**: 37-45.

[40]Li ST, Dai Q, Zhang SX, Liu YJ, Yu QQ, Tan F, et al. Ulinastatin attenuates LPS-induced inflammation in mouse macrophage RAW264.7 cells by inhibiting the JNK/NF-kappaB signaling pathway and activating the PI3K/Akt/Nrf2 pathway. *Acta Pharmacol Sin* 2018; **39**: 1294-1304.

[41]Harikrishnan H, Jantan I, Haque MA, Kumolosasi E. Anti-inflammatory effects of *Phyllanthus amarus* Schum. & Thonn. through inhibition of NF-kappaB, MAPK, and PI3K-Akt signaling pathways in LPS-induced human macrophages. *BMC Complement Altern Med* 2018; **18**: 224.

[42]Haque MA, Jantan I, Harikrishnan H, Ghazalee S. Standardized extract of *Zingiber zerumbet* suppresses LPS-induced pro-inflammatory responses through NF-kappaB, MAPK and PI3K-Akt signaling pathways in U937 macrophages. *Phytomedicine* 2019; **54**: 195-205.

[43]Qi G, Mi Y, Fan R, Li R, Liu Z, Liu X. Nobiletin protects against systemic inflammation-stimulated memory impairment *via* MAPK and NF-kappaB signaling pathways. *J Agric Food Chem* 2019; **67**: 5122-5134.